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RAP1 Is Essential for Silencing Telomeric *Variant Surface Glycoprotein* Genes in *Trypanosoma brucei*

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SUMMARY

Trypanosoma brucei expresses *variant surface glycoprotein* (*VSG*) genes in a strictly monoallelic fashion in its mammalian hosts, but it is unclear how this important virulence mechanism is enforced. Telomere position effect, an epigenetic phenomenon, has been proposed to play a critical role in *VSG* regulation, yet no telomeric protein has been identified whose disruption led to *VSG* derepression. We now identify tbRAP1 as an intrinsic component of the *T. brucei* telomere complex and a major regulator for silencing *VSG* expression sites (ESs). Knockdown of tbRAP1 led to derepression of all *VSGs* in silent ESs, but not *VSGs* located elsewhere, and resulted in stronger derepression of genes located within 10 kb from telomeres than genes located further upstream. This graduated silencing pattern suggests that telomere integrity plays a key role in tbRAP1-dependent silencing and *VSG* regulation.

INTRODUCTION

Trypanosoma brucei is a unicellular protozoan parasite that causes human African trypanosomiasis. In the bloodstream of its mammalian host, *T. brucei* periodically switches the major component of its surface coat, the variant surface glycoprotein (VSG; Barry and McCulloch, 2001), thereby evading immune elimination. Although there are more than 1000 *VSG* genes and pseudogenes in the *T. brucei* genome (Berriman et al., 2005; Marcello and Barry, 2007), *VSGs* can only be expressed from 1 of ~20 (Navarro and Cross, 1996) nearly identical *VSG* expression sites (ESs; Barry and McCulloch, 2001; Hertz-Fowler et al., 2008), which are polycistronically transcribed by RNA polymerase I (Pol I; Gunzl et al., 2003) and are located immediately upstream of telomeres (de Lange and Borst, 1982). In most ESs, the *VSG* is 0.2–1.6 kb upstream of the telomere DNA repeats, whereas the promoter is 40–60 kb upstream. Promoter-less *VSG* genes are also found on minichromosomes within 5 kb of telomeres, or on megabase chromosomes in gene clusters located at subtelomeric regions (Horn and Barry, 2005).

Mechanisms that ensure the monoallelic *VSG* expression remain elusive, even though several hypotheses have been proposed (Pays et al., 2004). One study showed that "silent" ES promoters are actually moderately active, but that transcription is rapidly attenuated, suggesting that transcription elongation is regulated (Vanhamme et al., 2000). Another study showed that only the active ES colocalizes with Pol I at an extranucleolar site, dubbed the expression site body (ESB; Navarro and Gull, 2001; Landeira and Navarro, 2007), suggesting that recruitment or limitation of a single ES to the ESB is crucial. Recent studies showed that depletion of a *T. brucei* homolog of the chromatin remodeling factor ISWI (TbISWI) elevated silent ES promoter activities without affecting *VSG* transcription (Hughes et al., 2007), and deletion of tbDot1B, a histone H3 methyltransferase, led to a 10-fold increase in silent *VSG* transcripts (Figueiredo et al., 2008), suggesting that chromatin modifications are important for ES silencing. Finally, because *VSG* is exclusively expressed from subtelomeric loci (de Lange and Borst, 1982), it has been proposed that the telomere structure plays an important role in *VSG* expression regulation (Horn and Cross, 1995; Horn and Barry, 2005; Glover and Horn, 2006; Dreesen et al., 2007).

Telomere-specific proteins are indispensable for telomere functions, which include protection of chromosome ends and maintenance of telomere lengths and the specialized telomere chromatin structure. Much is known about the mammalian telomere complex, which contains six core proteins (de Lange, 2005). Among these, TRF2 binds duplex telomere DNA and maintains chromosome end integrity. So far, tbTRF, a functional homolog of TRF2, is the only known *T. brucei* telomere-specific protein (Li et al., 2005). Another mammalian telomeric protein is RAP1, which does not contact DNA but is recruited to telomeres through its interaction with TRF2 (Li et al., 2000). In contrast, *S. cerevisiae* RAP1 is the predominant duplex telomere-DNA-binding factor (Shore, 1994). The difference between the DNA

binding activities of hRAP1 and scRAP1 can be explained by the fact that scRAP1 has myb and myb-like domains for DNA recognition whereas hRAP1 has a single myb domain with a negative surface charge on its third helix (Konig and Rhodes, 1997; Hanaoka et al., 2001).

ScRAP1 is essential for the telomeric heterochromatic structure, which can repress the transcription of subtelomeric genes, an epigenetic phenomenon termed telomere position effect (TPE; Gottschling et al., 1990). For genes targeted to subtelomeric loci, TPE is typically stronger at telomere-proximal than telomere-distal regions, spreading continuously inwards from telomeres (Renauld et al., 1993). However, TPE at native chromosome ends can be discontinuous, with a peak of repression at subtelomeric regions not immediately adjacent to telomeres (Pryde and Louis, 1999). TPE has also been observed in *T. brucei* and mammalian cells when reporter genes are analyzed (Glover et al., 2007; Glover and Horn, 2006; Gao et al., 2007; Pedram et al., 2006).

Many other proteins are involved in TPE, including the NAD^+-dependent histone deacetylases scSir2 and mammalian SIRT6 (Gasser and Cockell, 2001; Michishita et al., 2008), and yeast Ku70/80, which binds DNA ends (Mishra and Shore, 1999; Evans et al., 1998; Boulton and Jackson, 1998). The *T. brucei* Sir2 homolog, SIR2rp1, is also important for TPE (Alsford et al., 2007). Interestingly, the *P. falciparum* Sir2 homolog is essential for the telomeric heterochromatic structure and necessary for the monoallelic expression of subtelomeric *var* genes, which are critical virulence genes involved in antigenic variation (Freitas-Junior et al., 2005; Duraisingh et al., 2005). In *T. brucei*, however, there is no direct evidence supporting the idea that telomeres are important for monoallelic *VSG* expression. Depletion of tbTRF, telomerase, SIR2rp1, or tbKu80 had no effect on *VSG* expression (Li et al., 2005; Alsford et al., 2007; Glover et al., 2007; Janzen et al., 2004; Conway et al., 2002).

In this study, we report the identification of a novel *T. brucei* telomeric protein, tbRAP1, which interacts with tbTRF, associates with telomere DNA, and is essential for silencing ES-associated *VSGs*.

RESULTS

Identification of tbRAP1 as a tbTRF-Interacting Factor

We previously identified tbTRF as a telomeric protein that is essential for the telomere terminal structure but not *VSG* expression (Li et al., 2005). To identify additional telomere components, we carried out a yeast two-hybrid screen using tbTRF as bait. More than ten tbTRF-interacting candidates were identified, including the conserved hypothetical protein Tb11.03.0760. This protein has an N-terminal *BRCA1 C-t*erminus (BRCT) domain (Figures 1A and 1B), which is present in many proteins involved in DNA damage repair or cell-cycle checkpoints (Bork et al., 1997). Because RAP1 is the only known telomeric protein with an N-terminal BRCT domain (Li et al., 2000; Kanoh and Ishikawa, 2001; Park et al., 2002; Callebaut and Mornon, 1997), we compared this protein with known RAP1s more carefully. ClustalW analysis showed that this protein also has two central regions very similar to the myb and myb-like domains in scRAP1 (Figure 1), so we named this protein tbRAP1.

The sequence identity between tbRAP1 BRCT and other RAP1 BRCTs is 8%–18% (ClustalW), with the putative β1 β sheet being the most conserved region (Figure 1B; Zhang et al., 1998). The sequence homology is stronger within the myb and the myb-like domains, with identities of 18%–22% and 10%–23%, respectively (Figures 1C and 1D). Interestingly, both tbRAP1 and spRAP1 lack the C-terminal RCT protein-protein interaction domain (Figure 1A; Li et al., 2000), suggesting that tbRAP1 uses a different motif to interact with other proteins. Indeed, the N-terminal two thirds of tbRAP1 (amino acids 2–653) is sufficient and necessary for the interaction with tbTRF (Figure 2A). Compared with tbTRF self-interaction, the affinity of tbRAP1 to tbTRF is lower (Figure 2A). This is similar to the interaction between hRAP1 and hTRF2, which is weaker than hTRF2 self-interaction in a yeast two-hybrid assay (Broccoli et al., 1997; Li et al., 2000).

To confirm that tbRAP1 interacts with tbTRF in vivo, we carried out coimmunoprecipitation (coIP) using a rabbit antibody against tbTRF or a monoclonal antibody against the HA epitope tag, in cells containing a FLAG-HA-HA (F2H)-tagged tbRAP1 at its endogenous locus. In several independent experiments, we observed 3%–14% of F2H-tbRAP1 coIP with the endogenous tbTRF and nearly 10% of tbTRF coIP with the F2H-tbRAP1 (Figure 2B), confirming that tbRAP1 interacts with tbTRF in vivo, though weakly.

To confirm that tbRAP1 is a telomeric protein, we examined its subnuclear localization. Three *T. brucei* cell lines in which an endogenous tbRAP1 was tagged with an N-terminal Ty1, GFP, or F2H epitope were established. Indirect immunofluorescence (IF) was carried out with anti-tag monoclonal antibodies and an anti-tbTRF rabbit antibody. All the Ty1-, GFP-, and F2H-tagged tbRAP1 proteins partially colocalized with the endogenous tbTRF (Figure 2C; data not shown), which was confirmed by using an anti-tbRAP1 rabbit antibody (data not shown). These data suggest that tbRAP1 is localized at telomeres and possibly in other subnuclear compartments. This is similar to scRAP1, which targets 5% of the promoters in the yeast genome, in addition to telomere DNA (Pina et al., 2003).

To further confirm that tbRAP1 associates with the telomere, we carried out chromatin immunoprecipitation (ChIP) using a rabbit antibody against tbRAP1. We observed that telomere DNA was enriched in tbRAP1 and tbTRF ChIP (Figure 2D). In contrast, control DNAs, including the tubulin gene array and a random single-copy gene Tb11.0330, were not enriched in tbRAP1 or tbTRF ChIP under the same conditions, nor was telomere DNA enriched in any ChIP without formaldehyde crosslinking. TbTRF antibody precipitated more telomere DNA than tbRAP1 in normal ChIP (Figure 2D), which is consistent with the observation that all tbTRF but not all tbRAP1 seems to reside at telomeres.

TbRAP1 Is Essential for Normal Cell Growth

TbRAP1 double-allele knockout cell lines could not be established even though cells having one tbRAP1 allele deleted showed no growth defect (data not shown), suggesting that tbRAP1 is essential for cell growth. We therefore established inducible tbRAP1 RNAi cell lines: Ri-2 in VSG2-expressing SM cells (Wirtz et al., 1999) and Ri-9 in VSG9-expressing PVS3-2/OD1-1 cells (derived from SM cells). Both cell lines have a Ty1-tagged endogenous tbRAP1 allele and constitutively express the T7 RNA

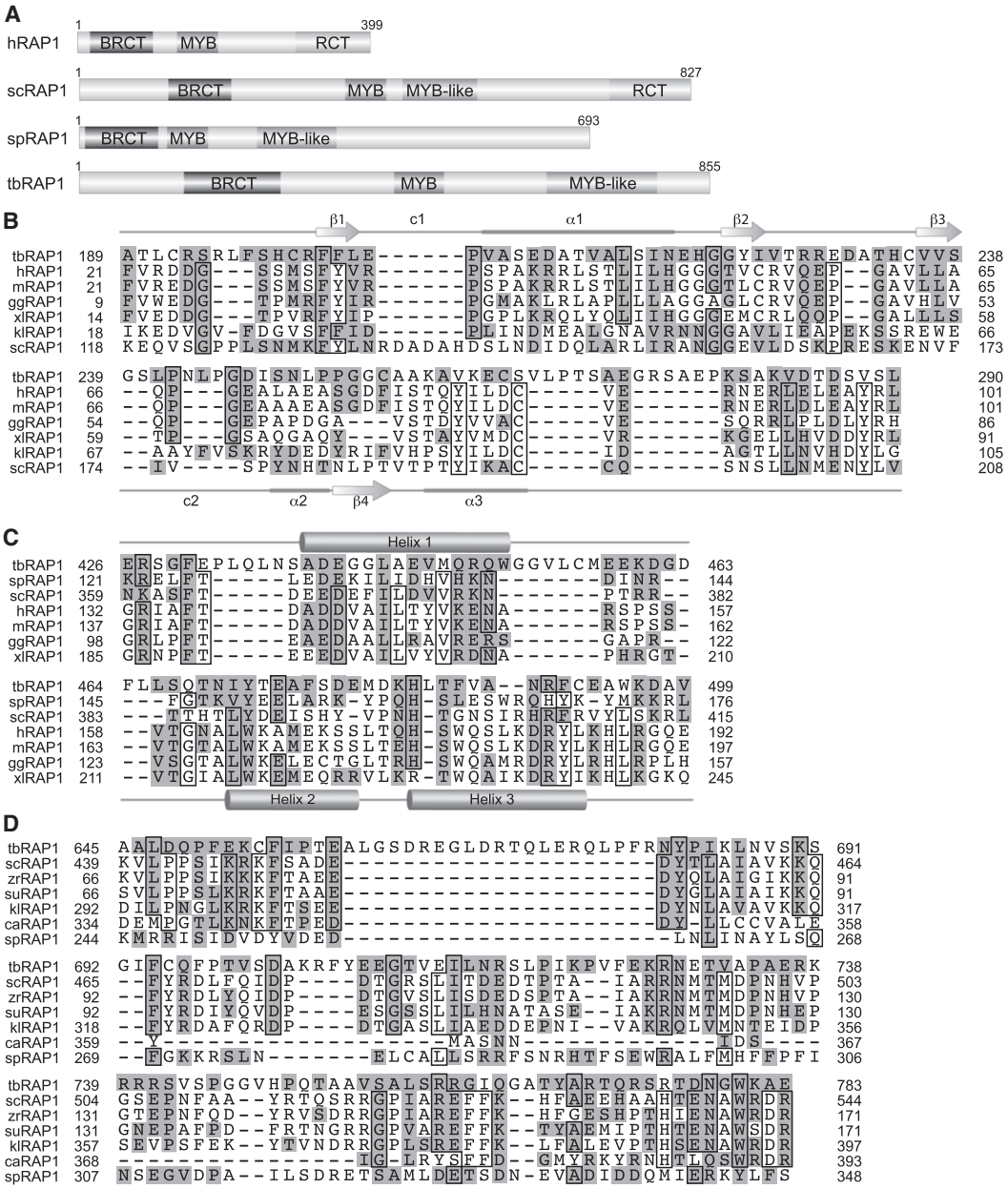


Figure 1. TbRAP1 Is Homologous to Telomeric RAP1 Proteins

(A) Domain structures for hRAP1, scRAP1, spRAP1, and tbRAP1. BRCT, myb, myb-like, and RCT (*RAP1 C-terminal*) domains are marked.

(B) Sequence alignment for RAP1 BRCT domains. The putative α helices and β sheets are marked as α1–3 and β1–4, respectively (Bork et al., 1997; Zhang et al., 1998). c1 and c2 are linker regions.

(C) Sequence alignment for RAP1 myb domains. The three putative helices are marked as cylinders above or below the sequences.

(D) Sequence alignment for RAP1 myb-like domains.

For (B)–(D), amino acids positions are indicated. Sequence similarities (Kyte and Doolittle, 1982) are shaded. Identical residues are boxed. ca, *Candida albicans*; gg, *Gallus gallus*; h, human; kl, *Kluyveromyces lactis*; m, mouse; sc, *Saccharomyces cerevisiae*; sp, *Schizosaccharomyces pombe*; su, *Saccharomyces unisporus*; tb, *Trypanosoma brucei*; xl, *Xenopus laevis*; zr, *Zygosaccharomyces rouxii*.

polymerase and the tet repressor. Ri-2 and Ri-9 are therefore isogenic but express different VSGs. After inducing tbRAP1 RNAi, western analysis showed that Ty1-tbRAP1 was greatly diminished by 12 hr and was undetectable by 36 hr (Figure 3A), indicating that knockdown of tbRAP1 was effective. Growth of these cells was arrested by ~48 hr after induction (Figure 3B), confirming that tbRAP1 is essential for normal cell growth.

We next examined whether depletion of tbRAP1 led to any telomere defect. To avoid nonspecific effects, tbRAP1 RNAi was induced for no more than 3 days, and three population

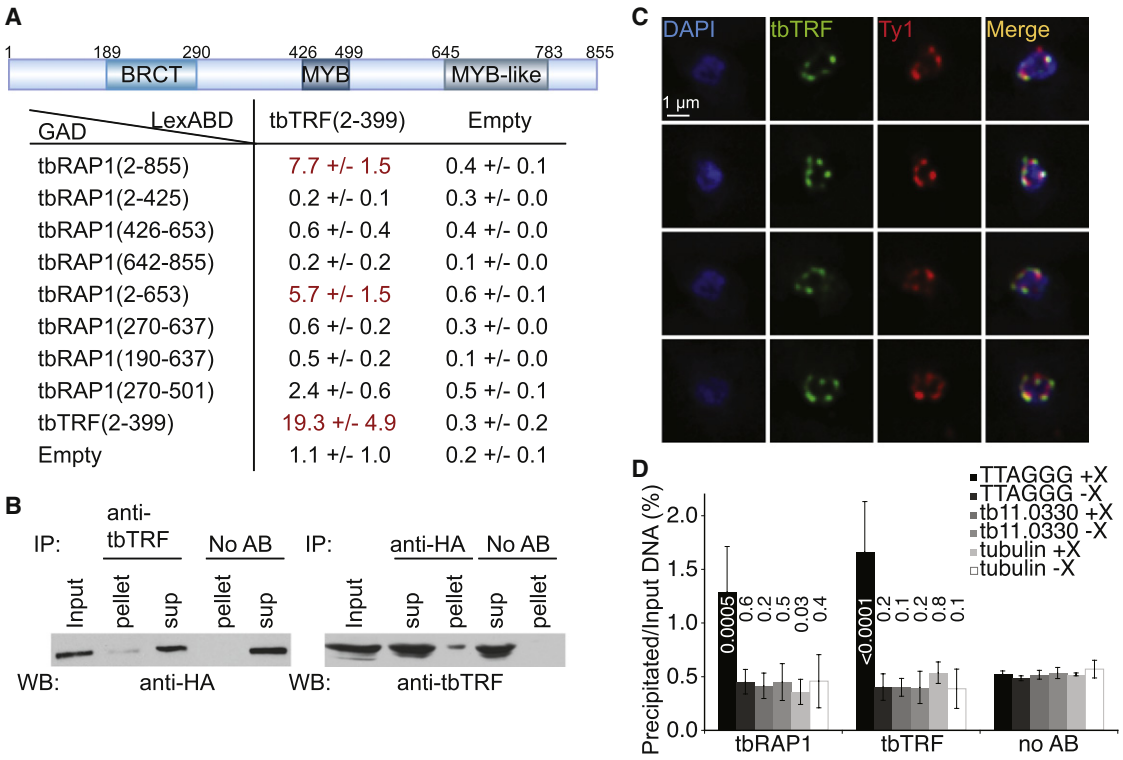


Figure 2. TbRAP1 Is a Telomeric Protein

(A) TbRAP1 interacts with tbTRF in yeast two-hybrid analysis. LexA binding domain (LexABD)-fused tbTRF and various Gal4 activation domain (GAD)-fused tbRAP1 fragments were used. Their interactions are shown as the average and standard deviation of β-galactosidase activities calculated from at least three independent assays. Positive results are highlighted in red.

(B) The endogenous F2H-tbRAP1 partially coIP with the endogenous tbTRF. In the left panel, an anti-tbTRF antibody 1260 (Li et al., 2005) was used for IP, and anti-HA monoclonal antibody 12CA5 was used for western blotting (WB). In the right panel, 12CA5 was used for IP and a chicken antibody against tbTRF was used for western blotting. Input (10%), sup (supernatant, 10%), and pellet (IP product, 30%) were indicated.

(C) Ty1-tbRAP1 partially colocalizes with tbTRF. Cells with an endogenous Ty1-tbRAP1 were stained with a rabbit antibody against endogenous tbTRF (green) (Li et al., 2005) and a monoclonal antibody (BB2) against Ty1 (red). DAPI was used to stain DNA (blue).

(D) Endogenous tbRAP1 associates with telomere DNA. ChIP was carried out either with (+X) or without (−X) formaldehyde cross-linking, using rabbit antibody against tbRAP1 or tbTRF, or, as a control, with no antibody. The average amounts of precipitated TTAGGG-repeat, tubulin, and Tb11.0330 DNA were calculated from three to seven experiments. Standard deviations are shown as error bars. Unpaired t tests were done to compare results from ChIP using tbRAP1 or tbTRF antibody with those using no antibody, and the resulting p values are listed over corresponding columns.

doublings (PDs) passed before growth was arrested. Telomere lengths were compared before and after the RNAi induction by Southern blotting, but no obvious telomere length changes were observed (data not shown).

TbRAP1 Is Essential for Silencing ES *VSGs*

Because scRAP1 is well known for its essential role in TPE (Gruntstein, 1997), we examined the possibility that tbRAP1 might play a role in *VSG* regulation.

The *T. brucei* Lister 427 strain contains 14 different *VSGs* in 15 ESs (Hertz-Fowler et al., 2008). We examined the expression of all 14 *VSGs* using quantitative real-time polymerase chain reaction (RT-PCR). To avoid effects from differences in mRNA stability and PCR efficiency, we compared mRNA levels for individual *VSGs* before and after induction of tbRAP1 RNAi.

In two independent Ri-2 lines (Table 1), all *VSG* mRNA levels (except *VSG2*) were increased 2- to 25-fold or 8- to 56-fold after 18 or 36 hr of tbRAP1 RNAi induction, respectively. At both time points, Ty1-tbRAP1 levels dropped to less than 6% of wild-type levels (Figure 3A). In contrast, *VSG2* expression exhibited a subtle decrease, and rRNA levels were stable (Tables 1 and 2). In addition to Pol I-transcribed *VSGs* and rRNA, we examined RNA polymerase II (Pol II)-transcribed tbTRF, tbTERT, and histone H4 (tbHH4), and found no more than 2-fold changes in their mRNA levels (Table 2). Because scRAP1 is also known to activate the expression of ribosomal protein and glycolytic protein genes (Pina et al., 2003), we also examined *tbRPS15* (Tb927.7.2370), a putative ribosomal protein gene, and *tbPGI* (Tb927.1.3830), which encodes glycosomal glucose-6-phosphate isomerase. There was no more than a 3-fold increase in the corresponding mRNA levels (Table 2), indicating that tbRAP1 is not necessary for their transcription. Finally, in parental cells lacking the tbRAP1 RNAi construct, the mRNA level for all tested genes exhibited less than 3-fold variation (Tables 1 and 2).

To determine whether *VSG*-derepression was specific to VSG2-expressors, we examined mRNA levels of various *VSGs*

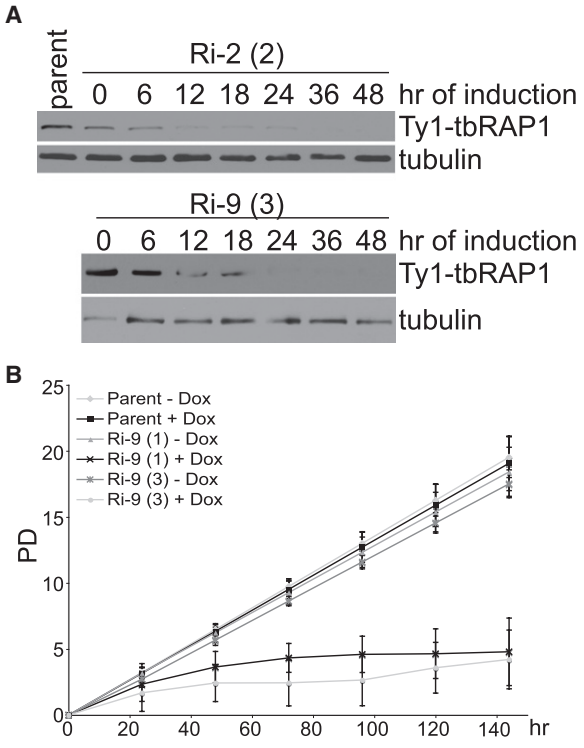


Figure 3. TbRAP1 Is Essential for Normal Cell Growth

(A) Induction of tbRAP1 RNAi resulted in efficient decrease in cellular tbRAP1 protein level. Whole cell lysates were prepared from Ri-2, clone 2 (top) or Ri-9, clone 3 cells (bottom). BB2 anti-Ty1 antibody was used for western blotting. Tubulin is shown as a loading control in this and following figures.

(B) Knockdown of tbRAP1 led to growth arrest. Growth curves were generated from five independent inductions. Average PDs against time are shown for two independent Ri-9 cell lines and their parent with (+) or without (−) doxycycline induction (Dox). Standard deviations are shown as error bars. A similar result was observed in Ri-2 cells (data not shown).

in the VSG9-expressing Ri-9 cell line. Northern analysis showed the same derepression for all tested *VSGs*, including *VSGs 2, 3, 11, 13, 15, 17, 18,* and *21* (Figure S1 available online). Therefore, the *VSG*-derepression induced by tbRAP1 knockdown is not specific to cells expressing a particular *VSG*. No *VSG* derepression was detected in tbTRF RNAi and control cells with empty RNAi vector (Figure S1A).

To determine whether a derepressed *VSG* in tbRAP1 depleted cells was transcribed at a similar level as when it is in an active ES, we used quantitative RT-PCR to compare the mRNA level of derepressed *VSG2* in Ri-9 cells to that of *VSG2* in Ri-2 cells. In Ri-9 cells, upon tbRAP1 knockdown, even when *VSG2* was maximally derepressed (~100-fold), its mRNA was still 70- to 100-fold less abundant than that in Ri-2 cells. Similarly, when a luciferase gene was located immediately downstream of an ES promoter, it had an activity of 3000–4000 units when the ES was active, but only 1–2 units when the ES was silent (after an in situ ES switch), and a maximum of 40 units when the ES was subsequently derepressed upon tbRAP1 knockdown. Therefore, the derepressed ESs were transcribed at an intermediate level between silent and fully active. This also means that in a silent ES, promoter-proximal and telomere-proximal genes are transcribed at ~0.3% and ~0.01% of their fully active levels, respectively.

Table 2. Depletion of tbRAP1 Does Not Affect Various Control Genes

Cells	hr	rRNA	tbTRF	tbTERT	tbHH4	tbRPS15	tbPGI
Ri-2(1)	0	1.0	N/A	N/A	N/A	1.0	1.0
	18	1.3/0.2	N/A	N/A	N/A	1.2/0.9	1.0/0.3
	24	1.3/0.3	N/A	N/A	N/A	1.1/0.5	1.8/0.7
	36	1.2/0.4	N/A	N/A	N/A	3.0/2.0	2.2/0.7
Ri-2(2)	0	1.0	1.0	1.0	1.0	N/A	N/A
	18	1.1/0.2	1.4/0.3	1.1/0.4	0.8/0.1	N/A	N/A
	24	1.2/0.2	1.7/0.9	1.4/0.7	1.2/0.7	N/A	N/A
	36	1.3/0.7	2.3/1.1	2.0/1.0	2.3/1.0	N/A	N/A
Parent	0	1.0	1.0	1.0	1.0	1.0	1.0
	18	0.8/0.2	1.1/0.3	1.1/0.4	0.8/0.1	0.5/0.3	0.8/0.7
	24	0.7/0.3	1.1/0.2	0.8/0.3	0.9/0.1	0.4/0.3	0.8/0.3
	36	0.7/0.1	0.8/0.4	0.6/0.3	1.0/0.1	0.9/0.6	1.1/0.2

Table 1. Depletion of tbRAP1 Resulted in Derepression of All Known ES *VSGs*[a]

Cells	hr	VSG2	VSG3	VSG6	VSG8	VSG9	VSG11	VSG13	VSG14	VSG15	VSG16	VSG17	VSG18	VSG19	VSG21
Ri-2(1)	0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	18	0.5/0.1	5.8/1.2	16.3/0.3	6.6/0.7	8.4/0.1	25.4/6.4	9.0/0.7	2.1/0.4	12.6/1.0	9.1/5.2	12.8/3.4	7.1/2.4	2.4/0.2	6.1/0.5
	24	0.5/0.1	7.1/2.8	22.7/13.4	8.4/2.8	10.9/3.5	29.8/1.9	11.9/5.6	1.9/0.1	12.4/0.2	9.7/6.1	14.1/4.0	5.4/1.7	14.5/7.4	9.5/2.0
	36	0.7/0.6	7.8/0.3	19.7/5.5	9.0/1.0	14.5/2.0	40.6/1.0	8.0/1.1	8.9/0.0	43.9/1.3	36.9/30.5	17.7/1.2	15.9/0.1	N/A	13.1/1.3
Ri-2(2)	0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	18	0.5/0.1	2.6/0.9	11.2/1.3	3.1/0.9	3.3/0.8	12.6/6.0	4.6/1.6	2.4/0.0	11.3/2.5	9.4/2.5	5.9/3.3	3.9/0.5	2.6/1.0	4.1/1.1
	24	0.5/0.1	6.5/2.2	40.3/35.2	8.9/0.8	9.4/3.8	23.4/7.3	8.3/2.3	2.8/0.2	16.4/0.3	9.1/4.8	24.4/13.9	5.2/1.7	5.5/0.9	8.7/0.2
	36	0.7/0.6	9.5/1.9	55.5/46.4	16.2/7.8	19.5/11.6	45.2/19.7	11.8/0.2	12.5/0.3	52.6/2.9	34.7/30.1	37.3/7.7	23.1/11.9	23.1/9.2	19.4/9.5
Parent	0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	18	1.1/0.5	1.8/0.1	1.6/0.8	1.2/0.2	0.7/0.1	1.6/0.1	1.1/0.4	1.1/0.2	1.2/0.2	1.4/1.2	1.0/0.3	0.7/0.3	N/A	1.4/0.2
	24	0.5/0.1	1.0/0.5	1.7/0.8	0.9/0.2	0.6/0.3	1.3/0.3	0.9/0.0	0.9/0.1	1.1/0.1	1.2/0.8	0.9/0.2	0.5/0.2	2.8/0.3	1.4/0.1
	36	1.0/0.6	2.8/1.3	1.8/1.2	1.2/0.3	1.0/0.3	2.2/0.1	1.3/0.4	1.2/0.0	0.9/0.1	1.8/1.1	1.2/0.5	0.6/0.1	1.3/0.3	1.5/0.6

[a] In both tables, the fold of increase in mRNA levels is shown as average/standard deviation calculated from quantitative real-time RT-PCR results using RNA samples collected at indicated time points from 3–7 independent inductions.

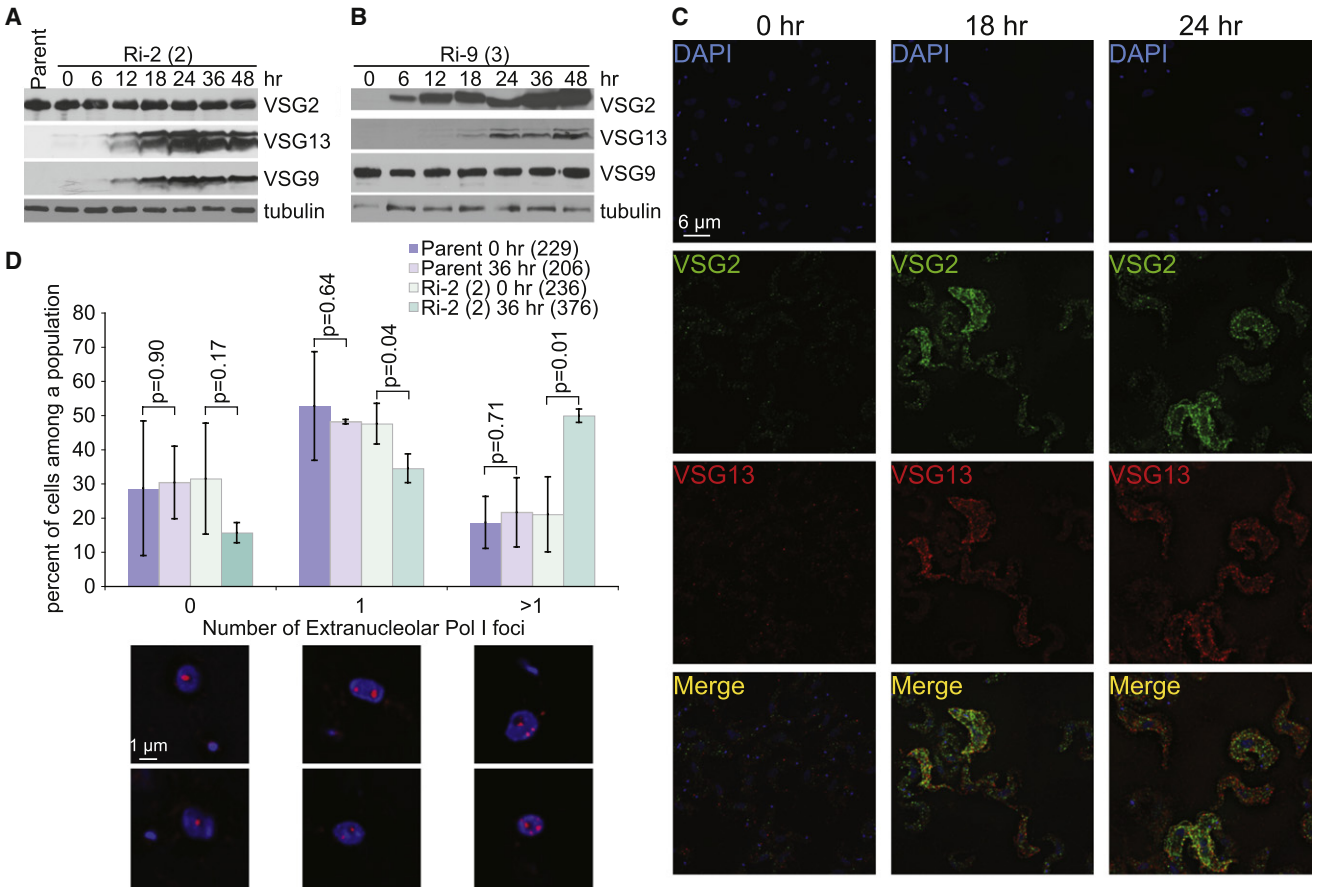


Figure 4. TbRAP1 Knockdown Resulted in Derepression of *VSGs* in Silent ESs and an Increase in the Number of Extranucleolar Pol I Foci

(A and B) Western analysis of derepressed VSGs. Whole cell lysates were prepared from Ri-2 clone 2 (A) or Ri-9 clone 3 (B) cells at various times after induction and probed with purified antibodies specific for VSG2, VSG13, or VSG9. The signal intensities of different VSGs are not comparable due to different affinities of various antibodies.

(C) IF analyses in Ri-9 clone 3 cells at indicated times after induction of tbRAP1 RNAi. Purified chicken antibodies specific for VSG2 (green) and rabbit antibody specific for VSG13 (red) were used. DNA was stained with DAPI (blue).

(D) TbRAP1 depletion led to a mild increase in the number of extranucleolar Pol I foci. The number of Pol I foci was determined by staining cells with Pol I antibody (red) (Navarro and Gull, 2001). Nucleoli were identified as regions that stain less with DAPI. Representative nuclei with 0, 1, or more extranucleolar Pol I foci are shown at the bottom. The percent of cells among a population falling into each category were scored in three independent experiments and the average and standard deviation (error bars) are indicated. Total number of examined nuclei is indicated next to the legend. P values are shown as results of unpaired t tests between induced or noninduced samples.

To test whether *VSG* derepression requires a promoter, we analyzed the expression of two promoter-less single-copy *VSGs* in both Ri-2 and Ri-9 cells. *VSG5* is at a nontelomeric locus (Horn and Cross, 1997), whereas *VSGG4* is at a subtelomeric locus on a minichromosome (Alsford et al., 2001; data not shown). Although primers for these two *VSGs* generated specific PCR products, their expression levels were not higher than background either before or after tbRAP1 knockdown (data not shown), and no *VSG5* or *VSGG4* mRNA could be detected in Ri-9 cells (Figure S1A). Therefore, tbRAP1 is only necessary for silencing ES-linked *VSGs* but not *VSGs* elsewhere in the genome.

TbRAP1 Deficiency Led to Simultaneous Expression of Different *VSGs* at the Surface of Individual Cells

To determine whether proteins were synthesized from derepressed *VSG* genes, we carried out western analysis using specific antibodies against VSGs 2, 9, and 13. In Ri-2 cells, low levels of VSG9 and VSG13 were detectable by 12 hr after induction whereas VSG2 was constantly expressed (Figure 4A). Similarly, in Ri-9 cells, VSG2 and VSG13 were detectable at 6 hr and 18 hr, respectively, whereas the expression of VSG9 remained constant (Figure 4B). In contrast, none of the silent VSG proteins were detectable in respective parent, TRF RNAi, or vector control cells (data not shown).

To determine whether multiple VSGs are expressed simultaneously in individual cells, we carried out IF analysis. In Ri-9 cells, the derepressed VSG2 and VSG13 were detected simultaneously on the cell surface (Figure 4C). Using other combinations of VSG-specific antibodies, we confirmed that VSG9 was constantly synthesized, and that the derepressed VSG2 and VSG13 were present in the same VSG9-expressing cells (data not shown). Although it was impractical for us to test whether

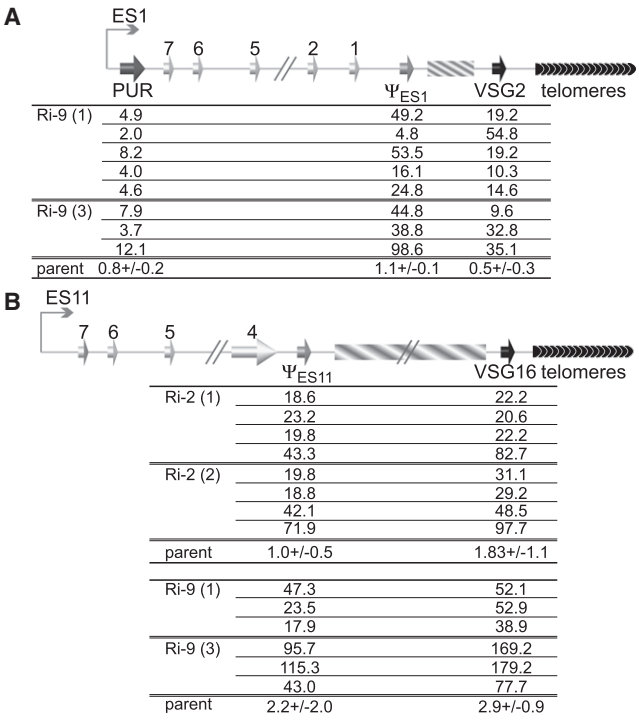
more than two VSGs were actually being synthesized simultaneously in individual cells, it is likely that they are.

Wild-type cells have a single active ES, which associates with the only one Pol I-enriched ESB (Navarro and Gull, 2001). Because multiple ESs are derepressed upon tbRAP1 depletion, we examined whether the subnuclear localization of Pol I is affected in Ri-2 cells by IF analysis using an anti-Pol I monoclonal antibody (Navarro and Gull, 2001). We observed that the fraction of cells with more than one extranucleolar Pol I foci increased from 20% to 50% after tbRAP1 RNAi was induced for 36 hr (Figure 4D). Interestingly, some of these foci seem to be much brighter than others, suggesting that different foci contain unequal amounts of Pol I.

To confirm that *VSG* derepression was specifically due to tbRAP1 depletion, we introduced an ectopic inducible F2H-tagged tbRAP1 into Ri-2 cells. Adding doxycycline to these cells led to a decrease in Ty1-tbRAP1 and an increase in F2H-tbRAP1 protein level but no detectable VSG9 or VSG13 (Figure S2) or any change in cell growth, indicating that the *VSG*-derepression phenotype resulted specifically from tbRAP1 deficiency.

Although tbTRF is not essential for *VSG* silencing (Li et al., 2005), it is possible that tbRAP1 depletion-induced *VSG*-derepression requires tbTRF function. To test this possibility, we established VSG2-expressing cell lines in which tbTRF and tbRAP1 could be knocked down simultaneously. Upon RNAi induction, VSG9 and VSG13 were still derepressed (Figure S3), indicating that tbTRF is not required for *VSG* derepression.

Depletion of VSG in *T. brucei* results in a cell-cycle arrest at the precytokinesis stage (Sheader et al., 2005). We questioned whether expression of multiple VSGs leads to a similar cell-cycle arrest. Cell-cycle analysis by fluorescence-activated cell sorting (FACS) showed that the fraction of cells in S phase is significantly reduced whereas the fraction in G2/M increased mildly after tbRAP1 depletion (Table S2). Therefore, tbRAP1 deficiency led to an altered cell-cycle profile that is different from what was observed in VSG-depleted cells.



Ri-9 (1)	4.9	49.2	19.2
	2.0	4.8	54.8
	8.2	53.5	19.2
	4.0	16.1	10.3
	4.6	24.8	14.6
Ri-9 (3)	7.9	44.8	9.6
	3.7	38.8	32.8
	12.1	98.6	35.1
parent	0.8+/-0.2	1.1+/-0.1	0.5+/-0.3

Ri-2 (1)	18.6	22.2
	23.2	20.6
	19.8	22.2
	43.3	82.7
Ri-2 (2)	19.8	31.1
	18.8	29.2
	42.1	48.5
	71.9	97.7
parent	1.0+/-0.5	1.83+/-1.1
Ri-9 (1)	47.3	52.1
	23.5	52.9
	17.9	38.9
Ri-9 (3)	95.7	169.2
	115.3	179.2
	43.0	77.7
parent	2.2+/-2.0	2.9+/-0.9

Figure 5. TbRAP1 Deficiency Led to a Stronger Derepression of Genes Located Closer to Telomeres

(A) A schematic diagram of the silent ES1 (top, not to scale) and the derepression levels of *PUR*, Ψ_{ES1}, and *VSG2* (bottom) after the tbRAP1 RNAi was induced for 24 hr in Ri-9 cells. The box with stripes represents the 70 bp repeats. Numbers indicate *ESAG* genes.

(B) A schematic diagram of the silent ES11 (top, not to scale) and the derepression levels of Ψ_{ES11} and *VSG16* (bottom) after tbRAP1 RNAi was induced for 36 hr in Ri-2 and Ri-9 cells. In both (A) and (B), the fold of increase in mRNA level for each gene was measured as described in Table 1. Results from several independent experiments were shown for Ri-2 and Ri-9 cells. The results for parental cells are shown as average and standard deviation calculated from at least three independent experiments.

TbRAP1 Knockdown Resulted in Greater Derepression of Genes Closer to Telomeres

TbRAP1 knockdown led to derepression of ES-linked silent *VSGs*, and the fold of increase in *VSG* mRNA should reflect the strength of tbRAP1-dependent silencing. In all ESs, *VSG* genes are located 0.2–1.6 kb from telomeres (Hertz-Fowler et al., 2008). As tbRAP1 is a telomeric protein, it is possible that tbRAP1-dependent silencing is stronger closer to telomeres. To determine how telomere-proximal and telomere-distal genes were affected after tbRAP1 depletion, we used quantitative RT-PCR to compare the derepression of unique genes in ES1 that are located at 60 kb (*PUR*, puromycin resistance), 7 kb (Ψ_{ES1}, a *VSG* pseudogene), and 1 kb (*VSG2*) upstream of the telomere (Figure 5A; Hertz-Fowler et al., 2008). Because independent inductions can give substantial variations, we used the same RNA samples for all three genes in each RT-PCR reaction and present individual experiment results. We found that *PUR* is always derepressed by a lesser amount (because, being close to the promoter, it is already somewhat transcribed in silent ES) than Ψ_{ES1} and *VSG2*, although Ψ_{ES1} was derepressed at a higher level than *VSG2* (Figure 5A). Our data indicate that tbRAP1 can silence the whole ES and more strongly closer to telomeres, albeit the silencing level might fluctuate at regions immediately upstream of the telomere. The graduated silencing pattern was confirmed by a similar analysis in ES11, which is silent in both Ri-2 and Ri-9 cells and has a unique Ψ_{ES11} (another *VSG* pseudogene) and *VSG16* located at 20 kb and 0.5 kb upstream of the telomere, respectively (Figure 5B; Hertz-Fowler et al., 2008). In both cell lines, Ψ_{ES11} was derepressed at lower levels than *VSG16* (Figure 5B), indicating that tbRAP1-dependent silencing is stronger at telomere-proximal regions.

DISCUSSION

TbRAP1 as an Intrinsic Component of the *T. brucei* Telomere Complex

Our data from yeast two-hybrid, coIP, IF, and ChIP showed that tbRAP1 interacts with tbTRF and associates with telomeres, though not exclusively, indicating that tbRAP1, like scRAP1, is a telomeric protein but also locates elsewhere in the genome

(Pina et al., 2003). No drastic telomere length changes were observed within three PDs when tbRAP1 RNAi was induced for 3 days, suggesting that tbRAP1 does not suppress rapid telomere length changes that have been observed in yeast, human, and *T. brucei* (Li and Lustig, 1996; Wang et al., 2004; van der Ploeg et al., 1984). However, we cannot exclude the possibility that tbRAP1 participates in telomere length control, because telomere length changes due to telomerase activity or the lack of it are very slow in *T. brucei* (3–6 bp/PD; van der Ploeg et al., 1984; Dreesen et al., 2005). A small change in three PDs (expected to be 9–18 bp), if there was any, would not be discernable in Southern analyses, when the average telomere sizes are ~10 kb.

The BRCT and myb domains are ubiquitous among RAP1 homologs (Teixeira and Gilson, 2005), but the function of RAP1 BRCT domain is unknown. The myb and the myb-like domains, at least in scRAP1, are required for DNA binding (Konig et al., 1996), as normally a minimum of two myb domains is necessary for DNA recognition (Ogata et al., 1994). Because tbRAP1 also contains a myb and a myb-like domain, it might bind telomere DNA directly. In addition, at least 13% of thymidines in *T. brucei* telomere TTAGGG repeats are replaced by the glucosylated base J in the bloodstream stage (Gommers-Ampt et al., 1991; van Leeuwen et al., 1996), so it will be interesting to determine whether tbRAP1 recognizes J-containing telomeres.

Regulation of Telomeric *VSG* Expression by tbRAP1

To our knowledge, tbRAP1 is the first telomeric protein identified in *T. brucei* whose depletion led to disruption of monoallelic *VSG* expression. All ES-linked *VSGs* were derepressed upon tbRAP1 knockdown, and this effect was not specific to cells expressing a particular *VSG*, suggesting that tbRAP1 is a critical ES transcription suppressor. It is possible that the *VSG*-silencing function of tbRAP1 might be independent of the telomere structure, because tbRAP1 is not exclusively at the telomere and the lack of obvious telomere length changes within a short period after tbRAP1 depletion. However, depletion of tbRAP1 did not affect the transcription of any tested Pol I- or Pol II-transcribed control genes or non-ES *VSGs*, indicating that tbRAP1 is not a general transcription regulator. In addition, tbRAP1-mediated silencing is stronger in regions within 10 kb of the telomere than those further upstream, suggesting that telomere structure is essential for this silencing.

The graduated strength of tbRAP1-dependent silencing is different in ES11 and ES1, indicating that silencing in different ESs are not identical. Similar differences have been observed in yeast, where TPE for subtelomeric reporter genes spreads continuously inwards from the telomere (Renauld et al., 1993), whereas TPE at native telomeres can have a peak of silencing not immediately upstream of the telomere (Pryde and Louis, 1999). In addition, different *VSGs* are derepessed at various levels upon tbRAP1 knockdown, which appears independent of *VSG* copy numbers or the distance between *VSG* and the ES promoter but reflects different levels of silencing in different ESs. Genome structure and chromosome context presumably can influence the degree of silencing. Further investigations will be necessary to determine which ES elements are influential.

In wild-type cells, silent ESs are transcribed at a low level for a short distance (Vanhamme et al., 2000), suggesting that the transcription elongation encounters an increasing antagonizing effect when it moves toward the telomere. TbRAP1 knockdown did not affect the active *VSG* but led to graduated derepression for genes along a silent ES, suggesting that tbRAP1-dependent silencing antagonizes transcription elongation from ES promoters (Figure 6A). Depletion of tbRAP1 lifted the silencing and the entire ESs including *VSGs* were transcribed (Figure 6B). However, derepressed ESs are transcribed at a 70- to 100-fold lower level than when they are fully active. Hence we propose that additional mechanisms are necessary to boost an ES to full activation (Figure 6C). This hypothesis is consistent with the observation that depletion of the chromatin remodeler tbISWI led to a partial promoter-proximal ES derepression (Hughes et al., 2007). Comparing silencing strength of ES-linked with that of non-ES reporter genes also suggested that a TPE-independent but ES-specific silencing is involved in *VSG* regulation, further supporting our hypothesis (Glover and Horn, 2006).

It has been proposed that concentrating a limiting amount of cellular Pol I transcription machinery at the ESB is an important mechanism to ensure monoallelic *VSG* expression (Navarro and Gull, 2001) and that, therefore, two forced fully active ESs have to switch back and forth rapidly and locate next to each other in the nucleus (Chaves et al., 1999). We observed an increase in the number of extranucleolar Pol I foci after tbRAP1

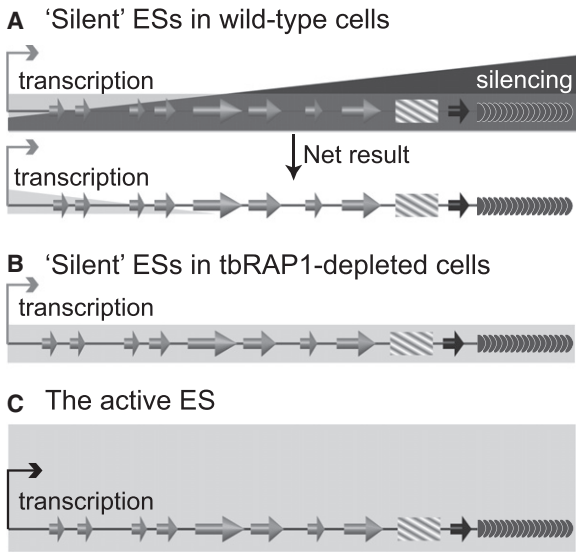


Figure 6. A Model for ES Silencing

ESs are similarly illustrated as in Figure 5. ES transcription is shown as a light gray bar. TbRAP1-dependent silencing is shown as a dark gray triangle, which is stronger at telomere-proximal regions.

(A) In wild-type cells, basal level transcription is initiated at silent ES promoters, but transcription elongation is quickly attenuated when transcription machinery moves toward the telomere and encounters an increasing level of tbRAP1-dependent silencing. The net result is a basal level transcription at the 5′ of silent ESs.

(B) When tbRAP1-dependent silencing is removed, transcription initiated at silent ES promoters can elongate throughout the ES and *VSGs* are transcribed at the basal level, which is maximally 100-fold higher than silent but still 70- to 100-fold lower than full activation.

(C) TbRAP1-independent mechanisms are necessary to fully activate an ES.

depletion, suggesting that a small amount of Pol I is available for multiple ES transcription at basal level.

Conclusions

Telomeres have been proposed to be involved in *VSG* regulation ever since the discovery that *VSGs* are expressed from subtelomeric loci (de Lange and Borst, 1982). Recent studies confirmed that TPE exists in *T. brucei* but also suggested that telomeres might not be essential for *VSG* silencing (Horn and Cross, 1995, 1997; Glover et al., 2007; Glover and Horn, 2006). We have identified tbRAP1 as a telomeric protein and showed that it is essential for complete ES *VSG* silencing. Our data strongly support the hypothesis that telomeres are important for the regulation of *VSG* expression.

In budding yeast, TPE depends on scRAP1, which binds to telomere DNA and recruits Sir proteins to establish a heterochromatic structure at telomeres (Grunstein, 1997). Recent studies indicated that silencing of subtelomeric *VSG* genes also depends on specific modifications of the chromatin structure (Figueiredo et al., 2008; Hughes et al., 2007). However, tbRAP1, but not SIR2rp1 or tbKu80, is essential for *VSG* silencing, indicating that tbRAP1-mediated silencing is not exactly the same as TPE in yeast. In addition, TPE in *S. cerevisiae* was studied using genes transcribed by Pol II (Gottschling et al., 1990), whereas in *T. brucei*, *VSGs* are transcribed by Pol I (Gunzl et al., 2003). It is possible that the chromatin structure affects transcription by different polymerases in different ways. Furthermore, in budding yeast, TPE affects initiation of gene transcription, where a promoter is immediately upstream of the reporter gene, whereas tbRAP1-mediated silencing seems to affect transcription elongation from ES promoters, which are 40–60 kb upstream of *VSGs*. These observations suggest that not all RAP1 homologs have identical functions and that further studies of tbRAP1 will be required to identify its precise roles in regulating *VSG* expression.

EXPERIMENTAL PROCEDURES

Plasmids

The full-length tbRAP1 was PCR amplified from *T. brucei* TREU927 genomic DNA and inserted into pLEW82-BSD (Li et al., 2005) together with an N-terminal F2H tag to generate an inducible tbRAP1 expression construct. The same fragment was inserted into p2T7-TABlue (Alsford et al., 2005) to make an inducible tbRAP1 RNAi construct. The subcloned full-length tbRAP1 was sequenced and the result submitted to GenBank (accession FJ597175). The hygromycin-resistance gene or *PUR* flanked by tbRAP1 5′ and 3′ untranslated region (UTR) were inserted into pBluescript SKII$^+$ to make two tbRAP1 deletion constructs. The phleomycin resistance gene, an α/β tubulin intergenic sequence, and the Ty1 tag (Bastin et al., 1996), together with flanking tbRAP1 5′ UTR and the N-terminal 500 bp of tbRAP1, were inserted into pBluescript SKII$^+$ to generate the tbRAP1 endogenous Ty1 tagging construct. F2H and GFP tagging constructs were similarly generated except *PUR* was used. Various fragments of tbRAP1 open reading frame were PCR amplified from *T. brucei* TREU927 genomic DNA and inserted into pACT2 (Clontech) or pBTM116 to make yeast two-hybrid constructs. The luciferase gene plus flanking 3′ and 5′ UTR were excised from pNS11 (Siegel et al., 2005) and inserted into pLF12 (Figueiredo et al., 2008) to generate pPUR-LUC.

Yeast Two-Hybrid Screen

The pBTM116-tbTRF-FL plasmid (Li et al., 2005) was transformed into yeast L40 strain (Vojtek et al., 1993), in which the expression of *lacZ* and *HIS3* are driven by minimal GAL1 and *HIS3* promoters fused to multimerized LexA binding sites. L40 cells harboring LexABD-tbTRF alone gave a mild transcription of *HIS3,* which was suppressed by 2.5 mM 3-aminotriazole. A GAD cDNA library generated with *T. brucei* 427 cDNA (Hoek et al., 2002) was used for the two-hybrid screen. A total of 1.6 million primary transformants were screened for *HIS3* and *lacZ* expression. GAD-fusion plasmids recovered from positive transformants were analyzed by restriction digestion. Independent clones were further tested to confirm that they do not give a positive result by themselves and that they interact with LexABD-tbTRF. Clones that passed all screens were sequenced and the insert identities were determined by searching the *T. brucei* genome database (Berriman et al., 2005). The interactions between different fragments of tbTRF and tbRAP1 were analyzed by liquid assays using ONPG as substrate.

T. brucei Cell Lines

The VSG9 Expressor Cells

The pPUR-LUC plasmid was transfected into VSG2 (also known as VSG221)-expressing SM cells (Wirtz et al., 1999) to generate Puromycin-resistant clones PUR-LUC1 (PL1). VSG9-expressing PVS3-2 cells were obtained by injecting PL1 cells into naive Charles River Strain CD-1 mice followed by isolation of in situ *VSG* switchers (confirmed by Southern blotting). To prevent further in situ switches in culture, the blasticidin-resistance gene was targeted immediately downstream of *VSG9* ES promoter to give rise to PVS3-2/OD1-1 cells, and these cells were maintained with 5 μg/ml blasticidin S.

Inducible tbRAP1 RNAi Cell Lines

Ri-2 cells were obtained by transfecting SM/Ty1-tbRAP1 cells with p2T7-TABlue-tbRAP1. Ri-9 cells were obtained by transfecting PVS3-2/OD1-1 cells with p2T7-TABlue-tbRAP1 and tbRAP1 Ty1-tagging constructs. All independent tbRAP1 RNAi clones behaved similarly upon induction with 0.1 μg/ml doxycycline.

Antibodies

Rabbit anti-VSG13, rabbit and chicken anti-VSG2 and the preparation of cross-reacting-determinant (CRD)-depleted sera were described in Figueiredo et al. (2008). Rabbit anti-VSG9 was kindly provided by Piet Borst, and CRD-depleted antibody was similarly prepared. Chicken antibody 606 was raised against a His_6-tbTRF expressed in bacteria. IgY proteins were first purified from egg yolks using EggStract kit (Promega) then affinity purified with His_6-tbTRF coupled CNBr-activated beads (GE). Rabbit antibody 597 was raised against a GST-$tbRAP1_{414-855}$ expressed from bacteria and purified with His_6-$tbRAP1_{414-855}$ coupled CNBr-activated beads.

Coimmunoprecipitation

A total of 200 million cells harboring an endogenous F2H-tagged tbRAP1 were lysed with two rounds of nitrogen cavitation in lysis buffer (50 mM Tris•Cl [pH 7.4]; 60 mM KCl, 1.5 mM $MgCl_2$, 0.4 M NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.01% SDS, 1 mM DTT). Protein extract was dialyzed against dialysis buffer (20 mM HEPES•KOH [pH 7.9], 0.2 mM EDTA, 0.2 mM EGTA, 100 mM KCl) and precleared with incubation with protein G beads (Sigma) prewashed with 1 × PBS/1% bovine serum albumin. The precleared lysate was used for IP using either 2 μg 12CA5 against HA (Rockefeller-Memorial Sloan Kettering Cancer Center Monoclonal AB facility) or 2 μl 1260 against tbTRF (Li et al., 2005). IP product was washed sequentially with buffer A (0.1% SDS; 1% Triton X-100; 2 mM EDTA pH 8.0; 20 mM Tris•HCl [pH 8.0]; 150 mM NaCl), B (same as buffer A but with 500 mM NaCl), C (0.25 M LiCl; 1% NP-40; 1% Na-deoxycholate; 1 mM EDTA pH 8.0; 10 mM Tris•HCl [pH 8.0]), and TE. All buffers are supplemented with 1 mM PMSF, protease inhibitor cocktail for mammalian cells (Sigma), 4 μg/ml pepstatin A, and 0.5 mg/ml TLCK immediately before use.

Reverse Transcription and Quantitative RT-PCR

Total RNA was extracted from cells using RNAstat (TEL-TEST, Inc) and purified with QIAGEN RNeasy kit and treated with DNase (QIAGEN). Reverse transcription was carried out using M-MLV (Promega) according to the manufacturer's protocol.

Quantitative RT-PCR was carried out using Bio-Rad iTaq SYBR Green Supermix with ROX according to the manufacturer's protocol. The amount of DNA was quantified by DNA Engine Opticon 2 (Bio-Rad). Sequences for

primers used in RT-PCR are listed in Table S1. Only primers giving specific PCR products were used. The normalized increase in mRNA level was calculated according to the following formula: ($mRNA_{V,\ n}/mRNA_{V,\ 0}$)/($mRNA_{T,\ n}/mRNA_{T,\ o}$), where $mRNA_{V,\ n}$ and $mRNA_{V,\ 0}$ represent the mRNA level for a particular *VSG* at n hr or 0 hr after tbRAP1 RNAi induction, and $mRNA_{T,\ n}$ and $mRNA_{T,\ o}$ represent the mRNA levels for β-tubulin at corresponding time points.

IF, ChIP, and FACS

IF was carried out as published (Lowell and Cross, 2004). ChIP was carried out as previously described (Li et al., 2005), with or without the formaldehyde treatment. The precipitated and input DNA samples were loaded onto a nylon membrane, hybridized with TTAGGG-repeat, tubulin, or Tb11.0330 probes, and quantified with a phosphorimager. The precipitated amount was calculated as a percentage of input material. Under both conditions, DNA was sonicated to an average size of 300–400 bp. FACS was carried out as previously (Li et al., 2005), except the results were analyzed with FlowJo (TreeStar). The cell-cycle profile was analyzed using the Watson Pragmatic template. Unpaired t tests were performed in Prism (GraphPad).

ACCESSION NUMBERS

The GenBank accession number for the subcloned full-length tbRAP1 reported in this article is FJ597175.

SUPPLEMENTAL DATA

Supplemental Data include three figures and two tables and can be found with the article online at http://cell.com/supplemental/S0092-8674(09)00085-3.

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